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Enhanced chiral inversion of *R*-ibuprofen in liver from rats treated with clofibric acid

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The chiral 2-arylpropionate anti-inflammatory drug ibuprofen is administered as a racemate. *In vivo*, the metabolic inversion of *R*-ibuprofen to the pharmacologically active *S*-enantiomer is mediated via a three enzyme pathway [1]. The initial formation of ibuprofen-CoA is stereospecific for the *R*-enantiomer and reportedly catalysed by microsomal long-chain fatty acid CoA ligase (EC 6.2.1.3) [2, 3]. Once formed the *R*-CoA thioester is either hydrolysed releasing *R*-ibuprofen or enzymically racemized. The *S*-CoA thioester thus formed is subsequently hydrolysed releasing the *S*-enantiomer. The identity and subcellular localizations of the racemase and hydrolase have not been established. In addition to a pivotal role in the overall inversion process formation of a CoA intermediate has led to incorporation of *R*-ibuprofen into adipose tissue triglycerides [4]. To date there is no information as to factors which may modulate either of these two metabolic pathways. Clofibric acid treatment induces a number of hepatic enzymes associated with fatty acid metabolism including the microsomal long chain CoA ligase [5] and various acyl-CoA hydrolases [6, 7]. In addition, administration of clofibrate causes a 2–3-fold increase in the total amount hepatic CoA [8]. It was conceivable therefore that clofibric acid treatment could modulate the chiral inversion of *R*-ibuprofen by virtue of either inducing both ligase and hydrolase activities and/or increasing synthesis of cofactor. In this context, the present investigation examined the effect of clofibric acid treatment on the *in vitro* chiral inversion of *R*-ibuprofen in rat liver homogenate.

Materials and Methods

The *R*- and S-enantiomer of ibuprofen (*R*, 97.3% and *S*, 98.4% purity) were obtained from the Boots Company (Australia). [1-^{14}C]Palmitic acid (50 mCi/mmol) was purchased from Amersham (Australia) and all other chemicals from the Sigma Chemical Co. Male hooded Wistar rats (250–300 g) were treated with either clofibric acid orally (Group I, 280 mg/kg/day, N = 5) or vehicle (Group 2, 5% w/v methylethycellulose) for 1, 2 or 5 days. Food and water were allowed *ad lib*. The livers were removed, perfused, homogenized with ice-cold buffer (10 mM phosphate containing 1.15% KCL, pH 7.4) and centrifuged at 700 *g* for 10 min. The supernatant was assayed for protein [9] and diluted to a final concentration of 30 mg/mL. A reaction mixture (2 mL) containing 150 mM Tris (pH 7.4), 6.2 mM $MgCl_2$, 0.05% Triton X-100, 2 mM EDTA, 1 mM dithiothreitol, 2.5 mM ATP, 0.6 mM CoASH, 6 mg homogenate protein and 0.2 mM *R*-ibuprofen was incubated at 37° and aliquots (100 μL) collected at various times (5–120 min) and analysed for *R*- and *S*-ibuprofen using a modified enantiospecific HPLC technique [10]. The microsomal fraction was prepared from the homogenate by differential centrifugation and long chain CoA ligase activity determined using [^{14}C]palmitic acid as previously described [11]. The statistical significance of the difference between groups was determined by either an analysis of variance or Student's *t*-test with Bonferroni correction [12].

Results and Discussion

Preliminary experiments established that the chiral inversion of ibuprofen was stereospecific for the *R*-enantiomer, dependent on the presence of ATP, CoASH, $MgCl_2$ and exhibited a pH optimum of 7.4. Omission of EDTA, dithiothreitol or Triton X-100 resulted in diminished inversion. In addition, inversion of *R*-ibuprofen was not observed in the presence of denatured hepatic protein. In all animals studied there was an initial rapid decrease in the concentration of *R*-ibuprofen which reached a maximum at 20 min. The *in vitro* system used was not constrained by lack of availability of cofactors as further addition of ATP and CoASH at that time produced no further formation of the *R*-CoA conjugate. It is conceivable that the CoA ligase was inhibited by either the reaction products of the initial step i.e. *R*-ibuprofen-CoA and AMP or adenosine [13]. Although a rapid decrease in the concentration of the *R*-enantiomer occurred this was not reflected by a corresponding quantitative increase in the *S*-enantiomer thus suggesting that the initial activation step was not rate limiting in the overall sequence of inversion. These results are in accord with the data of Knihinicki *et al.* [3] however in that study formation of the CoA conjugate was maximal at 10 min. Over the remaining 100 min the concentration of *R*-ibuprofen slowly increased, presumably due to hydrolysis of the *R*-CoA conjugate. In contrast, the concentration of the *S*-enantiomer continually increased such that at 120 min the total amount of *R*- and *S*-ibuprofen equated to the initial concentration of the *R*-enantiomer. Clofibric acid treatment for 1 day did not produce an increase in liver weight and there was no difference between the concentration time profiles for either *R*- or *S*-ibuprofen in liver homogenate from both the control and the treated animals (11.3 ± 0.1, 26.8 ± 1.6 and 11.9 ± 0.5 g, 26.1 ± 1.6%, liver weight and inversion, respectively). This was in marked contrast to the effects observed following administration of clofibric acid for either 2 or 5 days. Liver weight was markedly increased in Group 1 animals 13.48 ± 0.85 and 15.02 ± 0.6 g ($P < 0.001$) compared to Group 2 11.85 ± 0.35 and 11.45 ± 0.2 g for 2 and 5 days, respectively. A significant reduction (57%, $P < 0.01$) in the initial concentration of the *R*-enantiomer was observed after 20 min in the treated group when compared to liver homogenate from untreated animals (34%, Fig. 1A). This was a reflection of the increased catalytic activity ($P = 0.05$) of the microsomal long-chain fatty acid CoA ligase (Table 1). This enzyme has previously been implicated in the formation of both fenoprofen and ibuprofen-CoA and shown to be inducible by clofibric acid [2, 3, 5]. Once

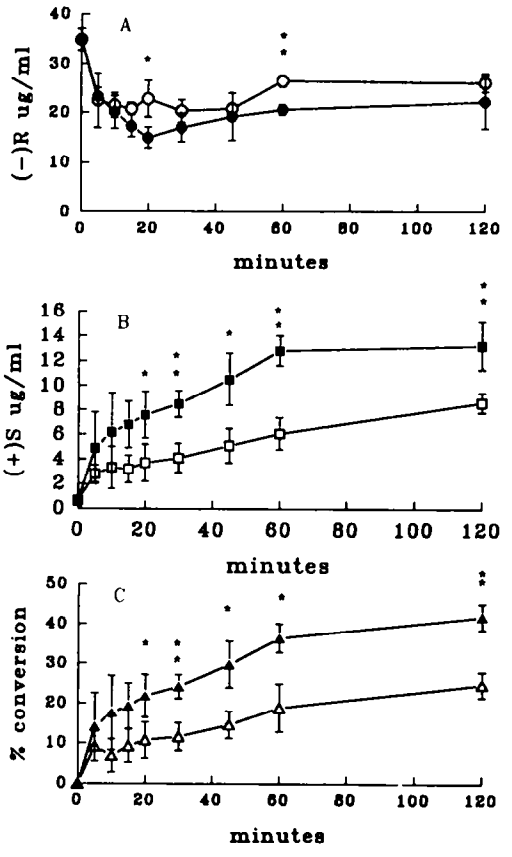

Fig. 1. Time course of ibuprofen enantiomer concentration and percent inversion in rat liver homogenate from 2 day clofibric acid treated animals (N = 5) (closed symbols) and untreated rats (open symbols). (A) and (B) *R*- (●, ○) and *S*- (■, □) ibuprofen concentration following incubation (37°) of rat liver homogenate (3 mg protein/mL) with 0.2 mM *R*-ibuprofen. (C) Per cent inversion (▲, △) of *R*- to *S*-ibuprofen in rat liver homogenate. Results are expressed as mean ± SD and statistically significant difference between groups denoted by * $P < 0.01$ and ** $P < 0.001$.

Table 1. Long chain CoA ligase activity in rat liver microsomes

Days	Group 1	Group 2
1	112 ±21.6	100 ±14.1
2	153.8 ±32.3	120 ±7.1
5	199 ±49	145 ±21.2

Rats were treated with clofibric acid (280 mg/kg/day) or vehicle (5% w/v methylethylcellulose) for either 1, 2 or 5 days. Hepatic microsomal long chain CoA ligase activity was determined using [^{14}C]palmitic acid as described [11]. Results (nmol palmitoyl-CoA/mg/min) are expressed as mean ± SD (N = 5). The effect of clofibric acid treatment was significant at a level of $P = 0.05$.

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formed the conjugate undergoes either racemization and hydrolysis releasing the *S*-enantiomer or hydrolysis producing unconjugated *R*-ibuprofen. This latter reaction was evident by the gradual increase in the concentration of the *R*-enantiomer subsequent to the formation of the conjugate. However, there was no difference between the quantity of *R*-ibuprofen released between 30 and 120 min in either group (5.2 and 5.8 μg/mL, Groups 1 and 2, respectively). It has been proposed that once formed the CoA conjugate is then either racemized spontaneously [14] or enzymatically [3]. Racemization is then followed by hydrolysis of the CoA thioester bond releasing *S*-ibuprofen. Incubation for a further period of 100 min resulted in the continued appearance of *S*-ibuprofen (Fig. 1B) such that at 120 min the metabolic chiral inversion of *R*-ibuprofen ranged from 24.8 ± 3.2% in the untreated animals to 41.9 ± 3.3% ($P < 0.001$) in the clofibric acid treated group (Fig. 1C). Although we were unable to delineate the individual effect of clofibric acid on either the activity of the racemase or hydrolase, it is apparent that there is induction of this composite step.

Previous studies have demonstrated that clofibric acid induces two hepatic cytosolic acyl-CoA hydrolases exhibiting different kinetic properties [7]. In addition hypolipidaemic drugs such as clofibrate differentially affect palmitoyl-CoA hydrolases, decreasing the activity of the microsomal enzyme with a concomitant increase in the activity of the cytosolic enzyme [6]. The preliminary results obtained in this study indicate that there may be differences in the hydrolysis of *R*- and *S*-ibuprofen-CoA thereby suggesting the involvement of two hydrolases which may be differentially affected by clofibric acid treatment. A similar profile for the induction of inversion was observed after clofibric acid treatment for 5 day, 37.3 ± 2.8% ($P < 0.01$) and 24.2 ± 0.6% Groups 1 and 2, respectively.

In conclusion, the metabolic chiral inversion of the 2-arylpropionates (2-APAs) involves the formation of the key intermediate *R*-2-APA-CoA. It is apparent from the data presented that formation can exceed hydrolysis and that this initial activation step is inducible by clofibric acid treatment. The potential pharmacological and toxicological ramifications of the increased availability of this intermediate include enhanced incorporation of ibuprofen into adipose tissue triglycerides [4], marked inhibition of endogenous lipid synthesis [15] and perturbations of cellular [acyl-CoA]/[CoASH] ratios. The broader implications of induction of xenobiotic-CoA formation requires investigation.

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Thiol-independent stimulation of soluble guanylate cyclase by NO-containing compounds

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NO-containing compounds are in clinical use for the treatment of coronary heart disease and arterial hypertension. Among these substances are the metal–nitroso complex sodium nitroprusside (SNP*), the acyl-sydnonimine molsidomine and various organic nitrates as glycerol trinitrate. Like the recently identified endothelium-derived relaxing factor [1] they induce vasorelaxation and inhibition of platelet aggregation by activating soluble guanylate cyclase (sGC, EC 4.6.1.2.), which catalyses the formation of cyclic GMP [2]. The active metabolites of the NO-containing compounds however, are still not defined. Thiols could mediate the generation of active metabolites of these compounds. Activation of sGC by organic nitrates requires the addition of specific thiols, preferably cysteine [3]. *S*-Nitrosothiols have been suggested as general active intermediates of NO-containing compounds [4, 5], though findings of others are contrary [6]. NO is another candidate [7, 8]. It is possible that various NO-containing compounds generate various active intermediates [9].

Thiols can influence the responsiveness of sGC for NO-containing compounds, since the enzyme is activated and inactivated upon oxidation with a concomitant loss of responsiveness [10–13]. The activation of sGC by SNP leads to an increase in the amount of reactive thiol groups of the enzyme [14]. Till now, no direct evidence has been given that NO-containing compounds can activate sGC in the absence of thiols. We studied the influence of the NO-containing compounds SNP and 3-(4-thiomorphlinyl)-*S*,*S*-dioxide-sydnonimine (C 78 0698) on a thiol free sGC preparation under anaerobic conditions (pO_2 below 6 Torr). This enabled us to demonstrate a thiol independent activation of sGC by these NO-containing compounds.

Materials and Methods

Materials. Reduced glutathione (GSH) and dithiothreitol (DTT) were purchased from Serva (Heidelberg, F.R.G.), C 78 0698 was from Cassella AG (Frankfurt, F.R.G.), SNP was from Merck (Darmstadt, F.R.G.).

Determination of guanylate cyclase activity. sGC activity was determined in a total volume of 0.1 mL with 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.1 mM [α-^{32}P]GTP (about 0.5 μCi per tube), 3.0 mM $MgCl_2$, 0.1 mg/mL γ-globulin and thiols when indicated. The reaction was started by the addition of the enzyme and was performed for 10 min at 16–18° (for technical reasons). Some experiments were performed at 37°. The reaction was stopped by the addition of 0.4 mL 120 mM zinc acetate, followed by 0.5 mL 120 mM sodium carbonate. The isolation of [^{32}P]cGMP and calculation of sGC activity was as described previously [15]. SNP and C 78 0698 were dissolved only prior to use. Incubations under reduced oxygen tension were performed in a glovebox. The glovebox and all solutions were evacuated and then gassed with nitrogen (99.9%) three times. pO_2 inside the glovebox and

* Abbreviations: SNP, sodium nitroprusside; C 78 0698, 3-(4-thiomorpholinyl)-*S*,*S*-dioxide-sydnonimine; sGC, soluble guanylate cyclase; DTT, dithiothreitol; GSH, reduced glutathione.